A MODEL SYSTEM FOR THE INVESTIGATION OF THE EFFECT OF SOIL PARTICLES ON THE MICROBIAL DECOMPOSITION OF HERBICIDES

FEBRUARY 1964
NO. 5

by

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Joint Highway Research Project

PURDUE UNIVERSITY
LAFAYETTE INDIANA

Progress Report

A MODEL SYSTEM FOR THE INVESTIGATION OF THE EFFECT OF SOIL PARTICLES ON THE MICROBIAL DECOMPOSITION OF HERBICIDES

TO: K. B. Woods, Director
Joint Highway Research Project

January 26, 1964

FROM: H. L. Michael, Associate Director
Joint Highway Research Project

Project: C-36-48
File: 9-5-2

Attached is a Progress Report on the "Chemical Weed Control" project. It is entitled "A Model System for the Investigation of the Effect of Soil Particles on the Microbial Decomposition of Herbicides" and has been authored by Mr. Walter Farmer who has been assigned to this project for the past several years. Mr. Farmer also utilized this report as his thesis for the Master of Science degree.

Mr. Farmer has also prepared a summary report of activities on this project to January 1964. This summary report was transmitted as the monthly progress report on January 25, 1964. As noted in that summary several of the field experiments are still active and continuance of this project is planned. Mr. Farmer will leave the project as of January 31 but will remain at Purdue and will be available to acquaint a new man with the active projects and areas for possible additional study.

The report is submitted for the record and will be transmitted to the Highway Commission and the Bureau for their review and comments.

Respectfully submitted,

Harold L. Michael

Harold L. Michael, Secretary

HLM:bc

Attachment

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Progress Report

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By
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Project: C-36-48
File: 9-5-2

Prepared as Part of an Investigation

Conducted by

Joint Highway Research Project
Engineering Experiment Station
Purdue University

in cooperation with

Indiana State Highway Commission

and the

Bureau of Public Roads
U S Department of Commerce

Purdue University
Lafayette, Indiana
February 26, 1964

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ABSTRACT

Farmer, Walter Joseph. M. S., Purdue University January, 1964. A MODEL SYSTEM FOR THE INVESTIGATION OF THE EFFECT OF SOIL PARTICLES ON THE MICROBIAL DECOMPOSITION OF HERBICIDES. Major Professors: Dr. M. M. Schreiber and Dr. H. W. Reuszer.

The purpose of this study was to develop a method for investigating the effect of soil constituents on microbial decomposition of herbicides which is applicable to several herbicides and the organisms capable of decomposing them.

Initial evidence of the microbial breakdown of the herbicide, 2,4-dichlorophenoxyacetic acid, was provided by the soil percolation apparatus. The effective organism, tentatively identified as a Pseudomonas sp., was isolated from the soil by the enrichment technique. The bacterial decomposition of 2,4-D was attributed to adaptive enzyme formation. Disappearance of the 2,4-D was followed by spectrophotometric analysis and growth of the bacterium by viable counts and turbidity measurements. Particles selected to simulate soil particles were kaolinite, Microbeads, Dowex 50W-X12 and Dowex 1-X8. These were washed by sedimentation and decantation and ranged in particle size from 8 to 149 μ in diameter.

Dowex 1-X8 adsorbed 2,4-D moderately up to the exchange capacity of the resin. None of the remaining particles demonstrated 2,4-D adsorption at pH 7.0. The bacterium was adsorbed by both Dowex 1-X8 and kaolinite. The presence of the particles had no effect on the rate of microbial decomposition of 2,4-D except with Dowex 1-X8. After 25 ppm 2,4-D was decomposed, there was a decrease in the rate when Dowex 1-X8 was present.

INTRODUCTION

As the popularity of herbicides as agents in weed control continues to rise, it becomes necessary to know the fate of these compounds when added to the soil. At least a part of all herbicides used eventually enters the soil. This includes those herbicides applied directly to the soil and those applied as a foliage spray. Little information regarding the length of time these materials will remain active in the soil is available and special attention is now needed to supply knowledge that will permit the intelligent selection of the type and rate of herbicide to use in different soil types and under varying conditions.

Farmers are vitally interested in the longevity of herbicides in the soil. Herbicides, when applied at rates less than those used for complete soil sterilization, are used for the selective control of weeds in agricultural crops such as corn, wheat, and alfalfa. Materials which accumulate in the soil from year to year may damage subsequent crops grown in that soil.

Highway departments, utility companies, and industrial concerns now use sterilants on rights-of-way, storage areas, along fence lines and around other structures.

The soil sterilants are applied directly to the soil for complete control of vegetation. Chemical weed control is used around these structures to reduce mowing and brush cutting costs. The use of the proper herbicide at the proper rate will bring about a further reduction in costs. Materials which will remain active in the soil for long periods reduce the number of retreatments required.

It was the purpose of this study to elucidate some of the factors affecting the length of time a herbicide is active in the soil. Generally speaking, soil micro-organisms are the most important agents in eliminating herbicides from the soil. Some of the soil constituents that influence microbial decomposition of herbicides are the clay particles. Knowledge of how herbicides interact with these materials will aid in the development of new herbicides.

More specifically, this study was designed to develop a model system to be used in studying the interaction of herbicides and soil factors. By adding varying concentrations of herbicide and clay particles in a controlled manner to a medium containing the specific organism responsible for the decomposition of the herbicide, quantitative information could be obtained concerning the factors responsible for the length of time a herbicide is active in the soil. The present problem was to obtain a specific organism which decomposes a certain herbicide

and to study the effect of adsorbing surfaces on the microbial decomposition. The herbicide, 2,4-dichlorophenoxyacetic acid (2,4-D) was chosen for this study because it is rapidly decomposed in the soil and its presence can be readily detected quantitatively by absorption spectrophotometry.

LITERATURE REVIEW

In recent years several investigators have presented evidence for the microbial decomposition of herbicides in the soil. Thorough reviews of the literature have recently been presented on this subject by Audus (1960), Alexander (1960), Bollen (1961), Freed and Montgomery (1963), and Sheets and Danielson (1960). Only a few pertinent articles which demonstrate the importance of microorganisms in herbicide decomposition will be cited here.

Factors such as soil moisture, pH, and temperature which favor bacterial growth have been shown to favor the disappearance of herbicides (Brown and Mitchell, 1948, and Sheets and Crafts, 1957). In addition, it has been found that microbial inhibitors such as sodium azide prevented the detoxication of herbicides (Audus, 1951). Autoclaving, which kills all bacteria in the soil, retarded the disappearance of 2,4-D (Brown and Mitchell, 1948) and phenyl ureas (Hill et al., 1955; Sheets and Crafts, 1957). The work of Audus (1949) using the soil percolation apparatus showed that 2,4-D was decomposed by microorganisms. He observed that 2,4-D disappeared from the soil solution only after a lag period which corresponded to

the time required for the microbial population to develop.

The final proof that microorganisms are capable of the decomposition of herbicides in the soil comes with the isolation of the effective organism and its growth with the herbicide as its sole source of carbon. Both Hill et al. (1955) and Douros and Reid (1956, 1957) isolated a Pseudomonas sp. which decomposed 3-(p-chlorophenyl)-1,1-dimethyl urea (monuron). The decomposition of 2,2-dichloropropionic acid (dalapon) is brought about by certain members of the genus Agrobacterium (Jensen, 1957). The herbicide studied most frequently has been 2,4-D and several organisms have been isolated which decompose this herbicide. Audus (1950) isolated an organism belonging to the Bacterium globiforme group which was responsible for 2,4-D disappearance. In 1952, Jensen and Peterson isolated two organisms designated Flavobacterium aquatile and a Corynebacterium sp. that decomposed 2,4-D in synthetic media. Later examination of F. aquatile by Weeks (1955) showed it to be a strain of Sporocytophaga congregata. Other investigators (Evans and Smith, 1954; Stapp and Spicher, 1954; Rogoff and Reid, 1956; Walker and Newman, 1956; Steenson and Walker, 1956; and Bell, 1957) have successfully isolated organisms that decompose 2,4-D.

Evidence for an induced enzyme system responsible for the disappearance of 2,4-D in the soil was provided

by the works of Steenson and Walker (1956), Audus (1960), and Bell (1960). Audus found that organisms such as B. globiforme and Nocardia sp. which could decompose 2,4-D in the absence of glucose rapidly lost this ability in the presence of glucose. Bell showed that there was a definite lag period before 2,4-D was oxidized by non-adapted cells whereas 2,4-D was oxidized immediately by adapted cells. Steenson and Walker found that cells grown in 2-methyl-4-chlorophenoxyacetic acid (MCPA) could oxidize 2,4-D whereas cells grown in peptone agar could not oxidize the 2,4-D.

More recently Alexander and Aleem (1961) used a mixed soil microbial population to follow the disappearance of 2,4-D. Their findings indicated that the time required for complete disappearance of 2,4-D was dependent upon the type of soil used. From these findings the question may be raised as to what factors are responsible for the 2,4-D disappearance. The disappearance of 2,4-D may have been due to the type of organism present. Another possibility would be that the organisms present were similar and that the differences in rate of 2,4-D disappearance may be attributed to other factors such as soil particles that affect the activity of the organisms.

As early as 1901, Whipple noted that water stored in small vessels produced more bacteria per milliliter than water stored in large vessels. He ascribed this to

the greater availability of oxygen in the small vessels. However, ZoBell and Stadler (1940) have shown that oxygen tension is not a limiting factor in the multiplication and respiration of aerobic bacteria in the ranges of 0.30 to 36 mg per liter. Heukelekian and Heller (1940) and ZoBell (1943) have both shown that when the supply of nutrient was limited, growth was stimulated by an increase in surface area.

Heukelekian and Heller (1940) found that when glass beads were present Escherichia coli grew in either glucose or peptone solutions as dilute as 0.5 mg per liter. Little growth occurred from 0.5 mg to 2.5 mg per liter in the absence of glass beads. Washed cultures of E. coli were inoculated into glucose and peptone media to give a final concentration of 170 bacteria per milliliter. The glucose and peptone concentrations were varied from 0.5 to 100 mg per liter in flasks of 250 ml capacity. To one series of flasks, four mm glass beads were added. The numbers of organisms were counted by the plate method after different periods of incubation.

ZoBell (1943, 1946) remarked that adsorption of nutrients on solid surfaces concentrated the nutrients for a bacterium which may subsequently come in contact with the solid surface either by adsorption of the bacterium on the solid surface or by random collison between the bacterium and the solid surface. In 1943, he

reported that porcelain, ignited sand, asbestos fibers, emery grit, and kieselguhr were beneficial to bacterial multiplication in dilute mineral solutions. Solid surfaces did not influence bacterial activity in dilute solutions of glucose, glycerol, or lactate whereas the beneficial effect was noted with increased solid surfaces in dilute solutions of sodium caseinate, lignoprotein, and an emulsified chitin preparation. In experiments with glass wool, glass beads, and glass tubing, he found that glucose, lactate, and glycerol were not adsorbed perceptibly while lignoprotein and emulsified chitin preparation were adsorbed. Bigger and Nelson (1941) found that an ignited talc, asbestos, kaolin, silica, unglazed porcelain, kieselguhr, and permutite and other inert solids increased bacterial growth in dilute nutrient solutions. Conn and Conn (1940) found that bentonite, kaolinite, beidelite, and illite stimulated bacterial activity which they attributed to increased surface area.

Working with the organisms, Pseudomonas sp. and Flavobacterium sp., Estermann and McLaren (1959) found an initial stimulation in the rate of ammonia production when lysozyme was adsorbed on kaolinite. This initial stimulation has also been observed with bentonite as an adsorbent and with the organisms Bacillus mycoides and Bacillus subtilis (Estermann, Peterson and McLaren, 1959). Estermann has attributed this to a concentrating effect. However, as ZoBell (1943) has pointed out, other factors such as adsorption exposing an active sight of the protein

to bacterial attack may be responsible for the enhancement. When kaolinite was present but the substrate was not adsorbed, the stimulating effect on ammonia production disappeared (Estermann and McLaren, 1959). These experiments were conducted at a lysozyme concentration of 1 mg protein per 2 ml of reaction mixture, a somewhat higher concentration than was used by other investigators who have reported beneficial effects of colloids.

Lamanna (1959) reported that beneficial effects of solid surfaces were generally observed at nutrient concentration less than 25 ppm. The work of Monod (1949) showed that with glucose this was the range in which nutrient concentration became limiting to growth. Particles larger than the bacteria tended to have a beneficial effect while particles smaller than bacteria tended to have either no effect or a negative effect on bacterial growth. ZoBell and Grant (1943) have reported that the rate of multiplication and oxygen consumption of marine bacteria in solutions containing less than 10 mg per liter is more or less directly proportional to the concentration of the substrate.

One of the beneficial effects of adsorbing surfaces may be due to the bacteria being adsorbed onto the surfaces. This adsorption may slow the diffusion of exoenzymes from the bacterial surface thus maintaining these enzymes in a position that would be more beneficial to

the bacteria (ZoBell, 1943).

The theory of bacterial adsorption has been supported by several studies. Using pure cultures, Rubentshick, Roisin and Bielansky (1936) showed that up to 98 percent of a 10 ml heavy bacterial suspension was removed by two grams of lake bottom mud. Maximum adsorption was obtained when the initial bacterial concentration was at 11 million bacteria per ml.

Gunnison and Marshall (1937) noted that the amount of adsorption differed with the type of organism used. Estermann and McLaren (1959) have reviewed the factors which have an effect on the adsorption of bacteria. The pH at which adhesion results has been found to be markedly influential. Gunnison and Marshall (1937) found the degree of adhesion of five organisms to be greater at pH 5.6 than at pH 7.2. The type of adsorbent is an important factor. Soils containing higher silt and clay percentages are efficient adsorbents for bacteria. The size fraction of an adsorbent has also been shown to be of importance. When the size of the adsorbent is reduced and approaches that of the bacteria, no adhesion takes place.

The activity of most herbicides also varies with soil composition (Sheets and Danielson, 1960). In a greenhouse experiment, the initial toxicity of 2,4-D was greater in sandy soils than in most clay soils (Crafts, 1949). Both amine salt and polypropylene ester formulations of 2,4-D

were adsorbed by montmorillinite, illite and kaolinite clays. The adsorption of 2,4-D increased as the cationic exchange capacity and specific surface increased (Hill, 1956). In both laboratory and greenhouse experiments, ethyl N,N-di-n-propylthiolcarbamate (EPTC) was adsorbed least by those soils in which it was most phytotoxic (Ashton and Sheets, 1959). Hill (1956) showed that the clay content, type of clay, and organic matter of soils influenced the amount of monuron adsorbed. Adsorption increased as clay content or organic matter increased.

As ZoBell (1943) has stated, "Much information on the factors which influence the metabolism of microorganisms could be gained by observing them in dilute nutrient solutions approximating the concentration found in their natural habitat." This points to the need for study of herbicides in dilute concentrations which more nearly approaches conditions as they occur in the field.

PART I. ISOLATION OF BACTERIUM

Methods and Materials

The herbicide, 2,4-D, used in this study was obtained in the acid form from Eastman Organic Chemicals. The acid was neutralized to pH 7.0 by titration with aqueous sodium hydroxide while stirred continuously with a magnetic stirrer.

A Chalmers silty clay loam soil from the Purdue Agronomy Farm, Lafayette, Indiana, was used to isolate the bacterium. Air-dried soil passing through sieve number 10 and retained on sieve number 20 was used.

The percolation apparatus used to obtain evidence of microbial decomposition of herbicides was a modification of the Lees and Quastel, 1946, method. An example of this apparatus is shown in Figure 1. Thirty grams of soil were placed in the upper portion of the flask and 250 ml deionized water containing 120 ppm of 2,4-D were added to the lower portion of the flask. The vacuum was regulated until percolation occurred at a moderate rate for 30 minutes to prevent water logging the soil. Circulation was then maintained at a rate of one drop per 15 seconds from the soil column. At this time, the water level on the flask was marked and fresh deionized water was added periodically

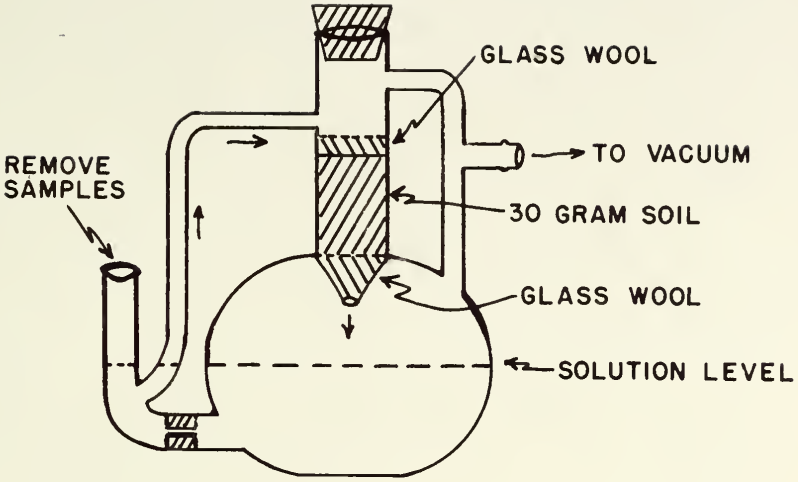

Figure 1. Percolation apparatus

to maintain this level. Room temperature was maintained at 25°C. A percolation apparatus containing only deionized water was used as a control. Five ml samples were removed every two to four days and analyzed spectrophotometrically for the presence of 2,4-D.

The herbicides, 2,4-dichlorophenoxy-α-propionic acid (2,4-DP), trichlorophenylacetic acid, and 2,4-dichlorophenoxy-σ-butyric acid 4-(2,4-DB) were also tested in the soil percolation apparatus using the same procedure as that used for 2,4-D. The concentration of the latter three compounds was initially 120 ppm.

The Beckman, Model DU, Spectrophotometer was used in the analytical analysis for 2,4-D using Pyrocell Quartz Absorption Cells of one centimeter light path. The absorption spectrum from 220 mμ to 320 mμ for 2,4-D was determined and its absorption maxima were established at 229 mμ and 283 mμ. These maxima were used in determining 2,4-D concentration.

Once it had been established that microorganisms were responsible for the herbicide decomposition, a bacterium was isolated using the enrichment technique and the shake flask method. Medium of the following composition designated as AL was used in the isolation procedure: .5 gram of ammonium nitrate, .8 gram of dibasic and .2 gram of monobasic potassium phosphate, .2 gram of magnesium sulfate, 0.03 gram of ferrous sulfate, .1 gram

of calcium chloride and .1 gram of 2,4-D in 1,000 ml of deionized water. The pH of the medium was adjusted to 7.3 before sterilization. Sterilization was accomplished by autoclaving at 121°C for 15 minutes. This was the medium used by Whiteside and Alexander (1960).

Results and Discussion

Evidence of Bacterial Decomposition of 2,4-D from Soil Percolation Studies

Evidence as provided by the percolation procedure for microbial decomposition of 2,4-D is shown in Figure 2. The herbicides, 4-(2,4-DB), 2,4-DP, and trichlorophenylacetic acid, were tested in the percolation apparatus in addition to 2,4-D for the purpose of determining if these herbicides were also subject to microbial decomposition. Such herbicides would be suitable for study in the model system which was developed in this research study. In order to obtain a more accurate comparison between herbicides, results were reported as the number of days required for 80 percent of the compound to disappear from the sample. The results obtained are reported in Appendix Table 1 and in Figure 3. The disappearance of 2,4-D and 4-(2,4-DB) is seen to follow the decomposition curve described by Audus (1951). This type of curve is typical of microbial action and indicates the importance of soil microorganisms in the disappearance of these herbicides from the soil. From

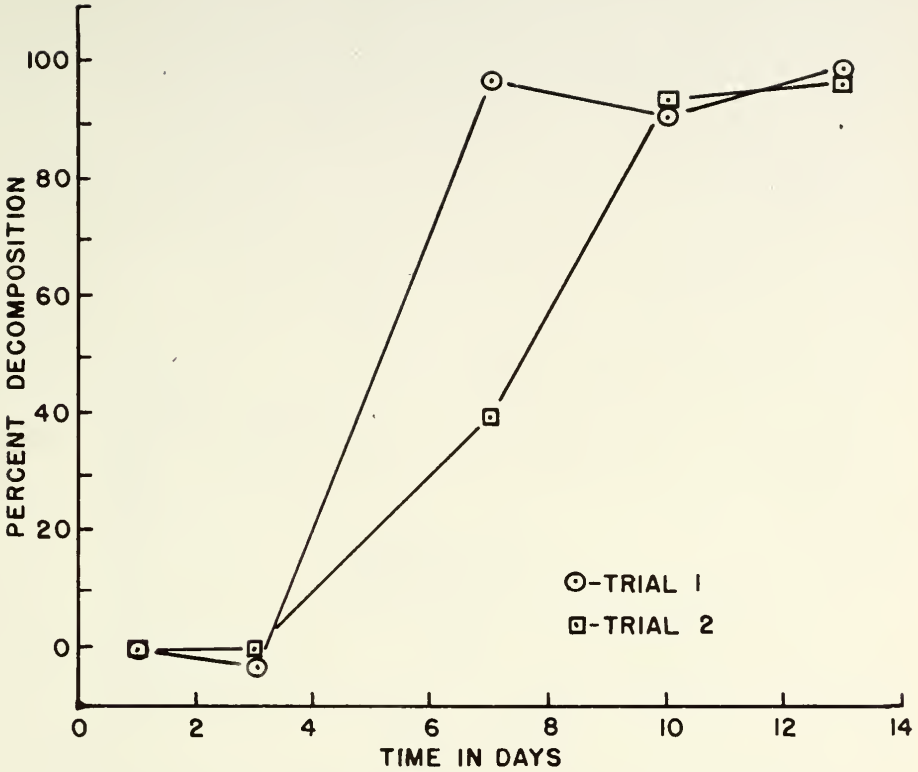

Figure 2. Decomposition of 120 ppm 2,4-D in the percolation apparatus

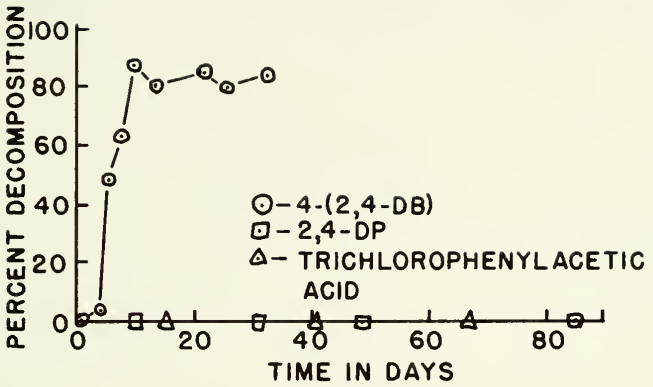

Figure 3. Decomposition of 120 ppm solutions of 4-(2,4-DB), 2,4-DP, and trichlorophenylacetic acid in the percolation apparatus

Figure 2, it may be noted that the decomposition was preceded by an initial lag period during which time the microbial population of the soil capable of decomposing the herbicide was increasing in number. This initial lag was followed by a period of rapid disappearance of the herbicide due to a large number of active organisms in the soil.

Trichlorophenylacetic acid and 2,4-DP showed no decomposition in the period of this experiment. This indicates that these materials are more resistant to microbial decomposition.

Isolation by Enrichment Technique

Having established that this soil contained micro-organisms active in the decomposition of 2,4-D, the enrichment technique was used to obtain an organism capable of decomposing 2,4-D in pure culture. A 500 ml flask containing 100 ml of the AL medium was inoculated with four grams of soil and incubated at 25°C on a Burwell, Model 40, wrist action shaker until UV analysis showed that the 2,4-D had been decomposed. A control flask containing the same medium minus the 2,4-D was inoculated with four grams of soil and incubated as above. After the 2,4-D had been decomposed, 1 ml of the medium was transferred to a fresh flask containing the above medium. This process was repeated each time the 2,4-D was decomposed. After six transfers, a loopful of the final medium was

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streaked onto agar of the same mineral composition as the AL medium plus 15 grams of agar per liter. Aseptic techniques were used throughout to ensure that the bacterium came from the soil. The inoculated agar plates were incubated at 25°C for five days until small, cream-colored colonies with entire edges were visible. Isolated colonies were removed with an inoculating needle and transferred into fresh flasks of medium with 2,4-D. Incubation of these flasks showed the bacterium to be capable of 2,4-D decomposition. The bacterium was carried through several single colony subcultures to insure having a pure culture. Microscopic observation and the Gram stain also indicated a pure culture.

PART II. CHARACTERISTICS OF THE BACTERIUM

Methods and Materials

The bacterium was classified by standard microbiological techniques.

Optimum growing conditions for the bacterium on liquid and solid media were determined using the media listed below.

The following medium was designated as X-1: 5 grams of peptone, 5 grams of glucose, 17 grams of agar, 0.1 gram of D L-methionine in 1,000 ml of deionized water.

The following medium was designated as ST: 5 grams of glucose, 1 gram of ammonium chloride, 1 gram of monobasic and 1 gram of dibasic potassium phosphate, .2 gram of magnesium sulfate heptahydrate, .1 gram of D L-methionine, 1 ml of A-Z solution, 17 grams of agar in 1,000 ml deionized water. The A-Z solution was composed of 0.022 gram of cupric sulfate pentahydrate, .1 gram of manganese chloride tetrahydrate, .1 gram zinc chloride, .01 gram of calcium nitrate hexahydrate, .02 gram of barium chloride dihydrate, .02 gram of $(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$ in 1,000 ml deionized water.

The following medium was designated as NA: 8 grams of nutrient broth, 15 grams of agar in 1,000 ml deionized water.

The following medium was designated as Czapek (CZA): 3 grams of sodium nitrate, 1 gram of dibasic potassium phosphate, .5 gram of magnesium sulfate heptahydrate, .5 gram of potassium chloride, .01 gram ferrous sulfate heptahydrate, 30 grams of sucrose, and 15 grams of agar in 1,000 ml of deionized water.

When broth media were desired, the agar was omitted from the above media.

The following medium was designated as RN_3: 2 grams of glucose, .3 gram of nutrient broth, .2 gram of yeast extract, .4 gram of monobasic and .4 gram of dibasic potassium phosphate, .2 gram of ammonium nitrate, .02 gram of magnesium sulfate, .001 gram of ferric chloride, .01 gram of calcium chloride, 1 ml of A-Z solution in 1,000 ml deionized water.

The following medium was designated as S-20YE: .6 gram of glucose, .03 gram of ammonium nitrate, .03 gram of dibasic and .005 gram of monobasic potassium phosphate, .002 gram of magnesium sulfate, .1 gram of calcium carbonate, .0005 gram of ferric chloride, .003 gram of yeast extract in 1,000 ml of deionized water.

The following medium was designated as MB_2: .2 gram of dibasic potassium phosphate, .3 gram of ammonium chloride, .01 gram of calcium chloride, .2 gram of magnesium sulfate heptahydrate, .001 gram of ferrous sulfate heptahydrate, 1 ml of A-Z solution and .1 gram of 2,4-D in 1,000 ml of deionized water. This was a modification of Bell's medium (1957).

The reactions of the above media were adjusted to pH 7.3 before autoclaving at 121°C for 15 minutes. When broth media were prepared, 100 ml of the broth were dispensed into 500 ml flasks and plugged with cotton previous to autoclaving. When agar media were prepared, 150 ml were dispensed into 250 ml flasks and plugged with cotton previous to autoclaving. Peptone blanks for serial dilutions were prepared by dispensing 9.4 ml of .1 percent peptone in 16 mm test tubes. During autoclaving, the peptone medium evaporated to give 9 ml. This had been

determined by previous experimentation.

Estimations of total bacterial counts were made with the Petroff Hauser Counting Chamber. Viable counts were made using the pour plate method. Bacterial growth was also estimated by turbidity measurements at 420 mμ on the Model DU Spectrophotometer. In the pour plate method, serial dilutions were made in .1 percent peptone blanks. Using 1 ml measuring pipettes, .1 ml aliquots of the appropriate dilution were transferred to each of eight four-inch petri dishes. Sixteen ml of melted agar cooled to 43°C in a constant temperature bath were added to each of eight plates. The plates were then swirled to insure thorough distribution of the bacteria on each plate. Plates were incubated at 25°C until they were counted. An average of the eight plate counts was then taken. For turbidity measurements samples were pipetted directly from the test flask into the cuvettes.

Stock suspensions of this bacterium for the above and subsequent experiments were maintained in broth medium MB_2 at 4°C. Transfers were made once a month to maintain the stock culture by inoculating a fresh flask of MB_2 at room temperature with 1 ml of the stock suspension and incubating on the wrist action shaker at 25°C for 48 hours before placing in the controlled temperature room at 4°C.

Further purification of the MB_2 medium was necessary due to the significant growth which occurred in the absence

of added 2,4-D for the growth rate studies. The purification procedure was accomplished by inoculation and incubation of MB_2 minus 2,4-D for 48 hours. The MB_2 medium minus 2,4-D was designated as MBB. Following incubation the medium was filtered through a sterilized millipore filter having pore openings of 0.45 μ. The millipore filter apparatus was sterilized by wrapping with brown paper and autoclaving for 15 minutes at 121°C. Sterilized 2,4-D was then added aseptically to bring the concentration of 2,4-D to .1 gram per liter. The filtered MB_2 medium was then dispensed into acid washed 500 ml flasks and plugged with gauze wrapped cotton plugs.

In all rate studies, the bacterial inoculum was prepared from 48 hour cultures by harvesting with centrifuging on the Servall Enclosed Super Speed Centrifuge, Type SS-4. Fifty ml polypropylene tubes with a SS-34 rotor having a maximum radius of 4.25 inches were used at a speed of 13,000 rpm for ten minute periods. The speed is equivalent to 20,200 times the force of gravity (Operating Instructions for Enclosed Superspeed Centrifuge, Type SS-4). The harvested cells were washed three times in fresh MBB medium and resuspended in MBB before use in the rate studies.

The spectrophotometric technique mentioned previously for detecting the presence of 2,4-D was refined for subsequent analysis. Four ml samples of the test medium

were centrifuged as described above in 12 ml reinforced glass centrifuge tubes with rubber adapters. Samples were then removed from the tubes while the tubes remained in the rotor by pipetting with a propipette into 14 mm test tubes. Samples were then read on the Model DU at 229, 252, 283, 290, and 340 mμ.

Results and Discussion

Morphology and Biochemical Characteristics

Standard microbiological identification techniques showed the microorganism to be a gram negative, motile rod 1.52 x .54 μ occurring singly or in pairs. It did not reduce nitrate nor hydrolyze starch or gelatin. It produced indole. It did not produce acid or gas from glucose and lactose. It turned litmus milk alkaline with reduction. Hydrogen sulfide was not produced and the organism could not utilize phenol or alkylamine. The organism contained catalase, was methyl red negative, and Voges-Prokauser negative. These characteristics resemble those of a Pseudomonas sp. as described by Skerman (1959). Electron microscopy, Figure 4, showed the presence of a single polar flagellum.

Maximum Growth on Liquid and Solid Media

The experiment was designed to determine which of the several solid media gave optimum conditions for viable counts. For this purpose, solid media were chosen which

Figure 4. Electron micrograph of 2,4-D decomposing bacterium

ranged in content from completely synthetic to complex media. A 48 hour culture of the bacterium in MB_2 served as the inoculum. A viable count was done on this inoculum using each of the several media as the counting base. These results are shown in Table 1. Plate counts were made 3, 5, and 7 days after inoculation. The number of colonies decreased with each consecutive counting apparently due to colonies which originally appeared as two or more fusing and appearing as one. No growth occurred on the CZA medium due to the inability of the organism to reduce nitrate to nitrite. Maximum number of colonies was obtained on the third day using X-1 agar as the counting medium. Therefore, the X-1 medium was used as the medium for future viable counts.

Another experiment was designed to determine which liquid media gave optimum conditions for bacterial growth. Again media ranging in content from synthetic to complex were used. After a 16 hour growing period of the bacterium in each of the liquid media, viable counts were made and results are shown in Table 2. As in the solid media determinations, the greatest growth was obtained when the medium contained a high proportion of complex components, i.e. peptone and yeast extract. This indicated that the bacterium required organic growth factors which it could not synthesize from glucose.

Table 1. Viable counts utilizing various agar media as the counting base at 3, 5, and 7 day incubation periods

Incubation period[1]	Media			
	CZA	NA	ST	X-1
Days	Number[2] of bacteria per ml x 10^{-6}			
3	0	135	140	165
5	0	130	139	155
7	0	123	138	152

[1]Period of time plates were incubated before counting.

[2]Average of 16 plates.

Table 2. Viable counts in various broth media at 16 hours

Media	Number[1] of bacteria per ml x 10^{-6}
X-1	118.0
ST	3.8
RN_3	113.0
S20-YE	24.6

[1]Average of 16 plates.

Studies of Bacterial Growth on 2,4-D

Growth Rate Studies. Two procedures, viable counts and turbidity measurements, were used for studying the growth rate of the bacterium on 2,4-D. In the first, viable counts were taken which showed a two-phase growth curve as depicted in Figure 5. During the initial growth phase showing exponential increase, there was no measureable decomposition of 2,4-D. During the second and much slower exponential increase, 2,4-D decomposition occurred. Since it was intended for 2,4-D to serve as the sole carbon source, attempts were made to decrease the initial exponential growth phase. When glass distilled water was used in place of the deionized water in the MB_2 medium there was no change in the duration of the exponential phase (Figure 6). In addition, when glass distilled water was used in the purification discussed earlier, there was no decrease in the initial phase (Figure 6). Growth of heterotropic organisms in distilled water has been reported by Garvie (1955) and Kalinenko (1957). Using the periods during which the bacterium is decomposing 2,4-D, growth rates were established. Figure 7 shows no change in the growth rate in the range of from 10 to 50 ppm 2,4-D. This indicates that a factor or a combination of factors other than 2,4-D concentration is growth limiting in this range. Monod (1949) showed that concentrations of glucose of less than 25 ppm were growth limiting to bacteria. Glucose is

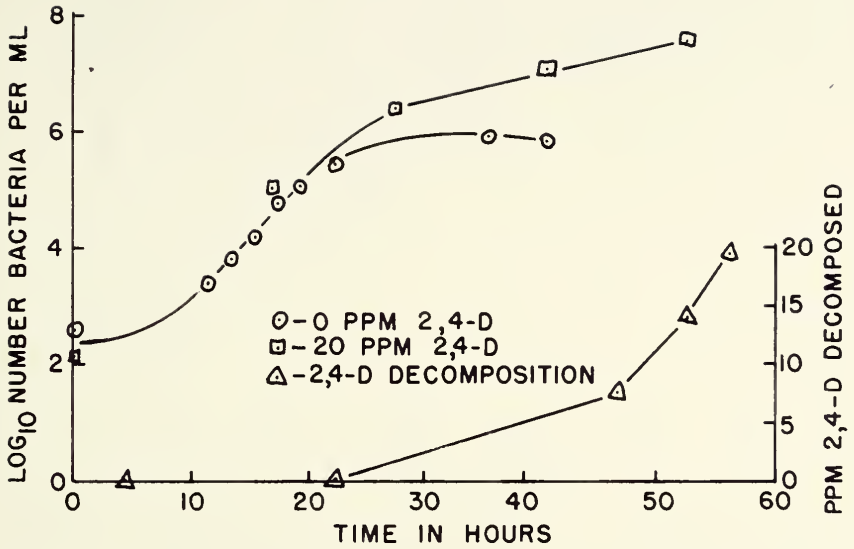


Figure 5. Growth curve of the bacterium in mineral salts medium prepared from deionized water

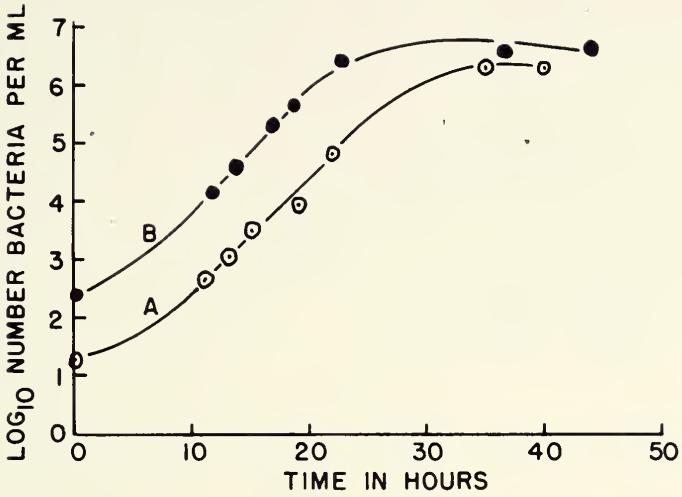


Figure 6. Growth curve of the bacterium in mineral salts medium prepared from A) distilled water and B) distilled water and purified as described in text

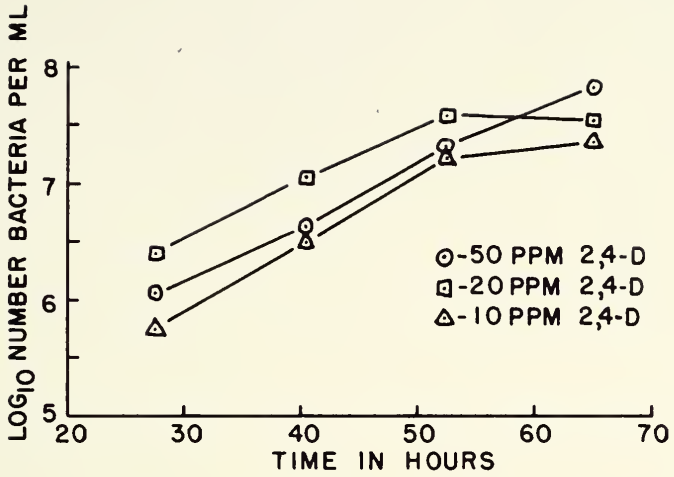


Figure 7. Growth rate of the bacterium in mineral salts medium containing 10, 20, and 50 ppm 2,4-D

a readily utilizeable carbon source and its uptake by bacteria was apparently the growth limiting step. When 2,4-D is the nutrient, it is metabolized at a slower rate than is glucose. Apparently some step in the metabolism of 2,4-D is growth limiting at 10 ppm or above rather than its rate of uptake by the bacterium. Growth rate studies were not made at concentrations lower than 10 ppm because spectrophotometric measurements of the rate of 2,4-D decomposition were not reliable at these concentrations.

Turbidity measurements at 420 mμ provided a rapid and less complicated procedure of comparing growth rates. Meaningful measurements required a high cell density which made the procedure less sensitive than viable counts to small increases in cell numbers. Examples of turbidity measurements will be shown in Figure 15. It should be noted that the use of high cell densities in turbidity measurements obscured the initial exponential growth phase of the bacterium which was observed using viable counts. Further results will be shown with the studies on the effect of soil particles on 2,4-D decomposition.

Growth Studies. Concentrations of 10 ppm and above of 2,4-D are much greater than those encountered under normal conditions in the use of 2,4-D as a weed control agent. The following experiment was designed to determine if this bacterium could decompose concentrations of 1 and

2 ppm which are similar to the concentrations found in field applications. Figure 8 shows that concentrations of 1 and 2 ppm of 2,4-D were decomposed by this organism. High concentrations of 2,4-D were inhibitory to the bacterium as shown in Table 3. However as stated previously, such concentrations would not be encountered in normal field applications.

Evidence for an Induced Enzyme System

The increased ability of 2,4-D treated soil to decompose 2,4-D over that of untreated soil may be due to either a selective or an adaptive process or a combination of both. Evidence that the ability of the bacterium used in this study to decompose is adaptive is shown in Figure 9. Cells which had been preadapted to 2,4-D by growth in MB_2 utilized 2,4-D immediately when placed in fresh MB_2. When the bacterium was grown in the absence of 2,4-D as in X-1 or RN_3 medium and then transferred to fresh MB_2, there was a considerable lag before 2,4-D was decomposed. Steenson and Walker (1956) showed similar evidence for the presence of an adaptive process present in their organism in the decomposition of 2,4-D.

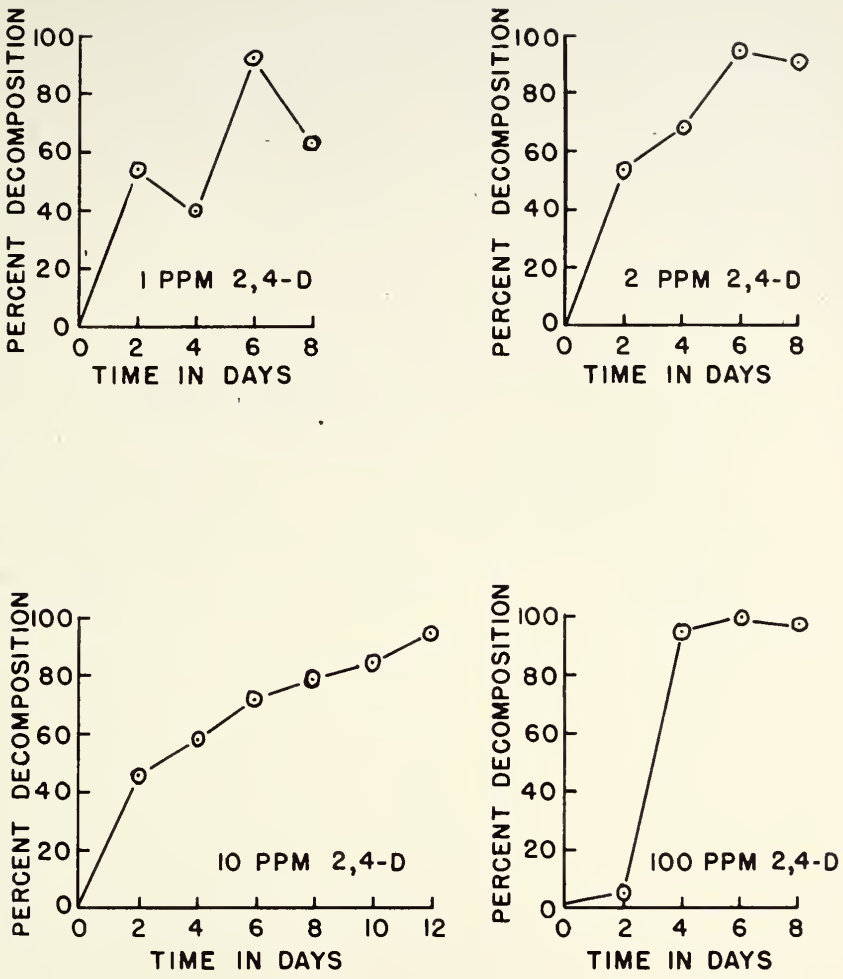


Figure 8. Bacterial decomposition of 2,4-D at low herbicide concentrations

Table 3. Bacterial decomposition of high initial concentrations of 2,4-D

Initial 2,4-D concentration	Maximum 2,4-D decomposed in 11 days	
ppm	ppm	percent
107	102	95.4
500	175	35.0
1,000	162	16.2
2,000	270	13.5
4,000	0	0.0

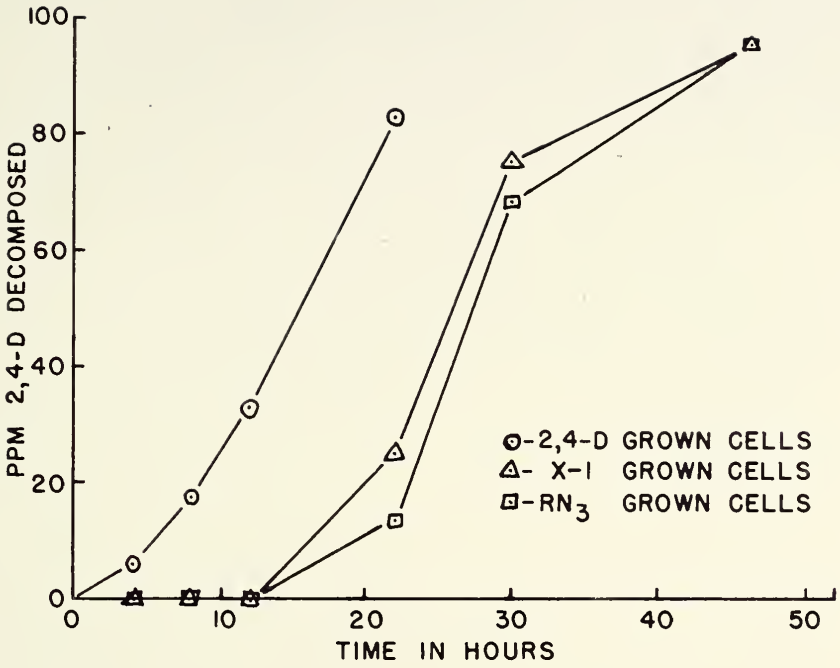


Figure 9. Decomposition of 2,4-D by heavy cell suspensions of adapted and non-adapted cells

PART III. ADSORPTION OF 2,4-D BY SOIL PARTICLES

Methods and Materials

The particles used to simulate soil particles were kaolinite, Microbeads, Dowex 50W-X12 cation exchange resin, and Dowex 1-X8 anion exchange resin. The kaolinite was obtained from Georgia Kaolin Research Laboratories, Elizabeth, New Jersey. It was sedimented according to Stokes' Law to give an equivalent spherical diameter of 8 to 40 μ. The calculations of Stokes' Law may be found in Appendix Table 2. The sedimentation was performed in a two liter beaker using a two percent clay suspension. The particles were suspended 20 times to insure the removal of all fine particles. Sedimentation was carried out in deionized water containing 3 meq. of sodium hydroxide per 100 grams of kaolinite to aid in the dispersion of the particles. After the final sedimentation, the kaolinite was washed with deionized water to remove excess alkali. The suspension was next titrated to pH 7.0 with sodium hydroxide. The particles were then allowed to sediment, the water decanted off, and the particles dried for 24 hours at 80°C. Finally, samples were removed from this sedimented clay for experimental use. The remaining particles were obtained in sieved form and microscopic

examination with phase contrast showed that there were no particles less than eight microns in diameter. The Microbeads were obtained from Microbeads, Inc. of Jackson, Mississippi. These were Class IV, Type 4,000, with a diameter from 8 to 37 microns. The ion exchange resins were obtained from the J. T. Baker Chemical Co. of Phillipsburg, New Jersey. Their diameters ranged from 74 to 149 microns. The particles were washed in deionized water by sedimentation and decantation to remove reagents used in the manufacture of the particles. The Dowex 50W-X12 was received in the acid form and was converted to the sodium form by treatment with sodium hydroxide. The Dowex 1-X8 was received and used in the chloride form. Excess surface alkali was removed from the Microbeads by treatment with hydrochloric acid. Suspensions of approximately 2.5 percent (w/v) were prepared in MBB with the three particles, Dowex 50W-X12, Dowex 1-X8, and Microbeads, and adjusted to pH 7.0. The actual concentrations of the suspensions were determined by drying a 5 ml aliquot in 20 ml beakers to a constant weight at 80°C. The beakers were cooled in a vacuum desiccator before weighing. An average of three determinations was used. Samples were pipetted from the particle suspensions while the suspensions were agitated constantly on a magnetic stirrer. These suspensions were diluted to two percent when used in further experiments.

The adsorption isotherms of 2,4-D on the particles were performed using 20 x 125 mm screw cap culture tubes. After the reagents were pipetted into the tubes, the tubes were capped and placed horizontally on the shaker at 25°C for periods varying from 15 minutes to 24 hours. At various times, tubes were removed from the shaker, contents were allowed to settle, and the supernatant was centrifuged and analyzed spectrophotometrically.

Results and Discussion

The adsorption isotherms of 2,4-D on kaolinite, Microbeads, and Dowex 50W-X12 were determined using two percent suspensions of the particles in concentrations of 2,4-D varying from 1 to 10 ppm. No adsorption occurred with these particles as shown in Table 4. Kaolinite and Dowex 50W-X12 possessed cationic exchange capacity and would not be expected to adsorb 2,4-D which is an anion at pH 7.0. Microbeads, which are composed of silica, would possess little or no exchange capacity.

Dowex 1-X8 is an anion exchange resin having an exchange capacity of 3.6 meq. per gram of dry resin. Adsorption isotherms of 2,4-D by this resin were run at resin concentrations of .01, .02, and .2 percent. Concentrations of 2,4-D varying from 10 to 18,000 ppm were used. The 2,4-D was moderately adsorbed by the anion exchange resin up to the exchange capacity of the resin as shown in Figure 10. The adsorption was not complete in 15 minutes.

Table 4. Adsorption of 2,4-D by particles

Particle	Initial ppm 2,4-D supernatant	Final ppm 2,4-D supernatant
mg kaolinite/ml		
20.0	2.0	2.1
20.0	5.0	5.0
20.0	10.0	10.0
20.0	20.0	20.0
mg Dowex 50W-X12/ml		
21.5	1.0	1.1
21.5	2.5	2.5
21.5	5.0	5.0
21.5	10.0	10.0
mg Microbeads/ml		
20.8	1.0	0.9
20.8	2.5	2.5
20.8	5.0	5.1
20.8	10.0	10.0

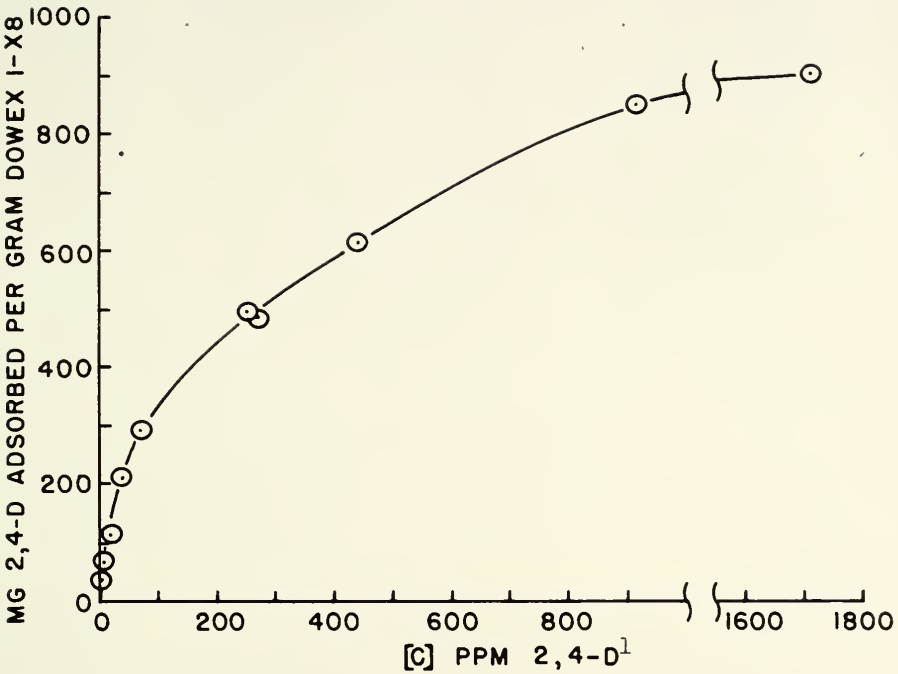


Figure 10. Adsorption isotherm of 2,4-D on Dowex 1-X8

[1]The abscissa shows the concentration of 2,4-D remaining in solution after equilibrium with the resin was attained.

However, very little adsorption occurred in 24 hours over that which occurred in one hour.

In order to determine the bacterial decomposition of 2,4-D in the presence of Dowex 1-X8, it was necessary to desorb the 2,4-D from the resin for spectrophotometric analysis. Sodium sulfate, hydrochloric acid, sodium chloride and a mixture of hydrochloric acid and sodium chloride were used to replace the 2,4-D at the active sites on the resin. Solutions of .342 normal final concentrations of the desorbing agents were used. Table 5 shows that the chloride anion was more effective in replacing 2,4-D than was an equivalent amount of the sulfate anion. The acidic chloride solution was used in later experiments in order to stop bacterial action during the replacement process.

It was necessary to run an adsorption isotherm of 2,4-D on Dowex 1-X8 in the presence of the acidic chloride solution. This curve, Figure 11, was used as a standard curve for the quantitative determination of 2,4-D in the presence of the resin.

Table 5. Comparison of desorption of 2,4-D from Dowex 1-X8 by the chloride and sulfate ion

Desorbing agent	pH	Percent 2,4-D in supernatant
chloride	2.5	45
sulfate	3.0	24

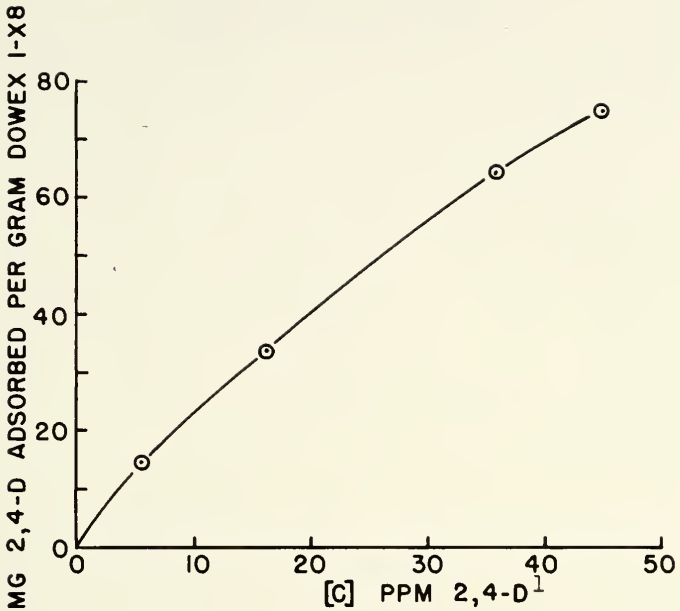


Figure 11. Adsorption isotherm of 2,4-D on Dowex 1-X8 in presence of 0.342 N chloride ion at pH 2.5

[1]The abscissa shows the concentration of 2,4-D remaining in solution after equilibrium with the resin was attained.

PART IV. EFFECT OF SOIL PARTICLES ON MICROBIAL DECOMPOSITION

Methods and Materials

In order to evaluate any effect of the particles on the microbial decomposition of 2,4-D, it was necessary to know if the bacterium was adsorbed by the particles. This was done by inoculating 1 ml of a heavy bacterial cell suspension, prepared as in Part II above, into 100 ml of the two percent particle suspension in MBB. A flask which contained no particles was inoculated as a control. The flasks were placed on the shaker at 25°C for 15 minutes and then allowed to stand for five minutes after which time viable counts were made on the supernatant from each flask.

For the experiments on determining the rate of 2,4-D decomposition, flasks were prepared containing two percent suspensions of the particles in MB_2. These flasks were then autoclaved for 15 minutes at 121°C. Control flasks were prepared which contained no particles. The flasks were then inoculated with a heavy, washed bacterial cell suspension prepared as in Part II above. Beginning 15 minutes after the initial inoculation, 8 ml samples were removed periodically and placed in 14 mm test tubes. Particles were allowed to settle for five minutes at which

time 3 ml were removed from the test tubes for turbidity measurements and 4 ml were removed and analyzed for 2,4-D as described in Part II above. Aseptic techniques were used throughout. Appropriate controls were run for each aspect of the experiment.

Results and Discussion

Bacterial Adsorption by Soil Particles

Kaolinite and Dowex 1-X8 were found to adsorb from 17.9 to 56.7 percent of a heavy cell suspension as shown in Table 6. These two particles were selected for this experiment as they were representative of a surface possessing a negative charge and one possessing a positive charge respectively. In the case of kaolinite, it can be seen that the percent adsorption of the bacterium decreased as the number of bacterial cells increased. This indicated that the number of sites on the kaolinite available for bacterial adsorption were being saturated. Rubentshick, Roisin, and Bielansky (1936) found that the percent adsorption of bacteria by a lake bottom mud increased with increasing initial bacterial numbers up to 11 million bacteria per ml. The percent adsorption decreased thereafter.

The Effect of Particles on 2,4-D Decomposition

The presence of kaolinite, Microbeads, and Dowex 50W-X12 had no effect on the rate of uptake of 2,4-D by the

Table 6. Bacterial adsorption by soil particles

Particle		Number of bacteria per ml		Percent adsorption
	g/100 ml	initial	final	
kaolinite	1.0	7.78×10^3	4.93×10^3	36.6
kaolinite	2.0	5.37×10^7	4.41×10^7	17.9
kaolinite	2.0	1.55×10^8	1.27×10^8	18.0
Dowex 1-X8	1.2	9.12×10^7	3.94×10^7	56.7

bacterium as measured in this study. The rate of 2,4-D uptake in the presence and absence of particles is shown in Figures 12 and 13. There are two reasons for expecting the absence of an effect on the rate of 2,4-D uptake. Evidence has been presented that a concentration of 2,4-D in the range of 10 to 100 ppm is not a determining factor in the rate of reproduction of the bacterium. Lamanna (1959) found that for the presence of solid surfaces to be beneficial, nutrients must be present at concentrations less than 25 ppm. This is the range of glucose concentration which Monod (1942) found to be rate determining. The concentration at which 2,4-D becomes rate determining is apparently less than 10 ppm. The herbicide, 2,4-D, is commonly used at rates less than 10 ppm. Thus it is possible that the presence of inorganic particles has an effect on the rate of decomposition under normal field conditions.

A second reason for not expecting an effect of the particles on the rate of 2,4-D utilization was that the herbicide was not adsorbed by kaolinite, Microbeads or Dowex 50W-X12. ZoBell (1943) found that it was only substances which were adsorbed onto solid surfaces that were more readily utilized in the presence of these solid surfaces.

From the results of the experiments on the bacterial adsorption by particles, a beneficial effect of particles

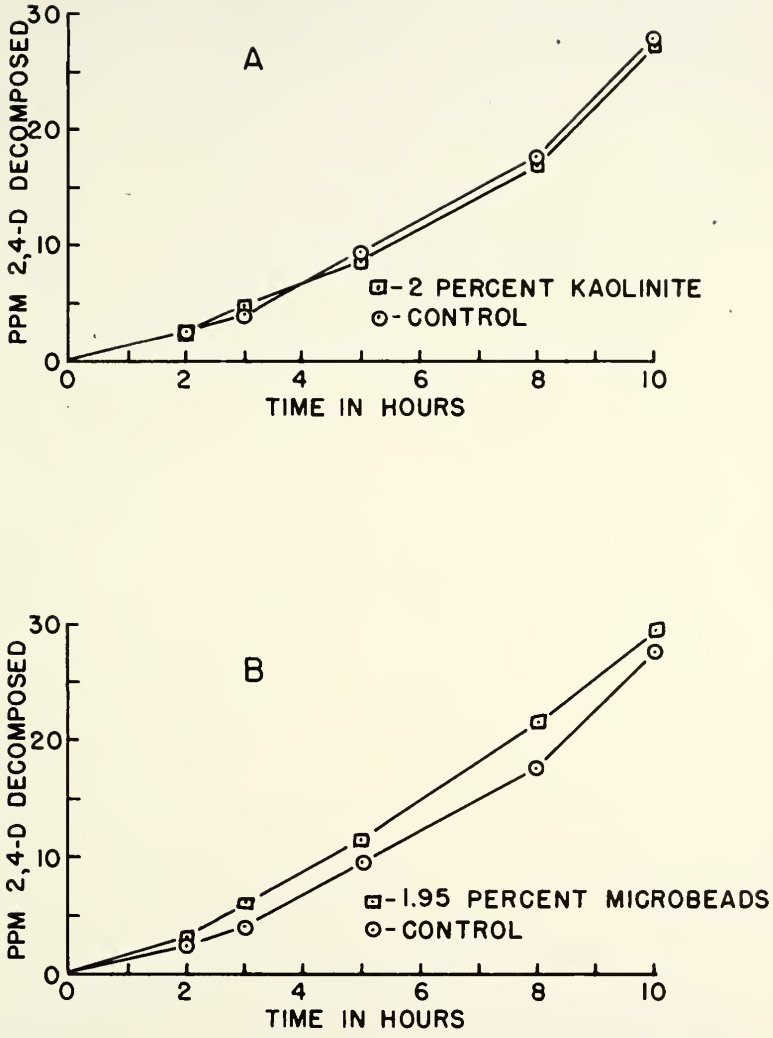
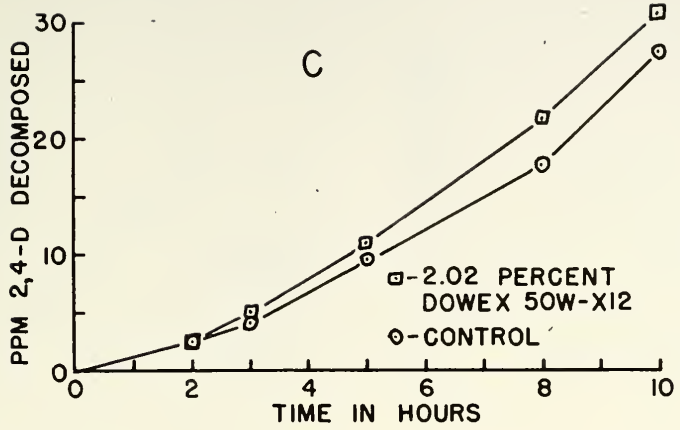
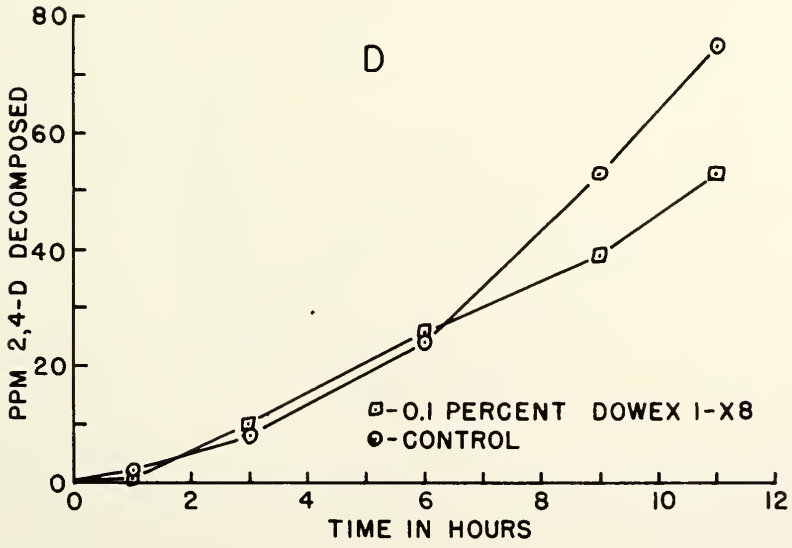


Figure 12. Effect of particles on bacterial decomposition of 100 ppm 2,4-D

Figure 12. (continued)





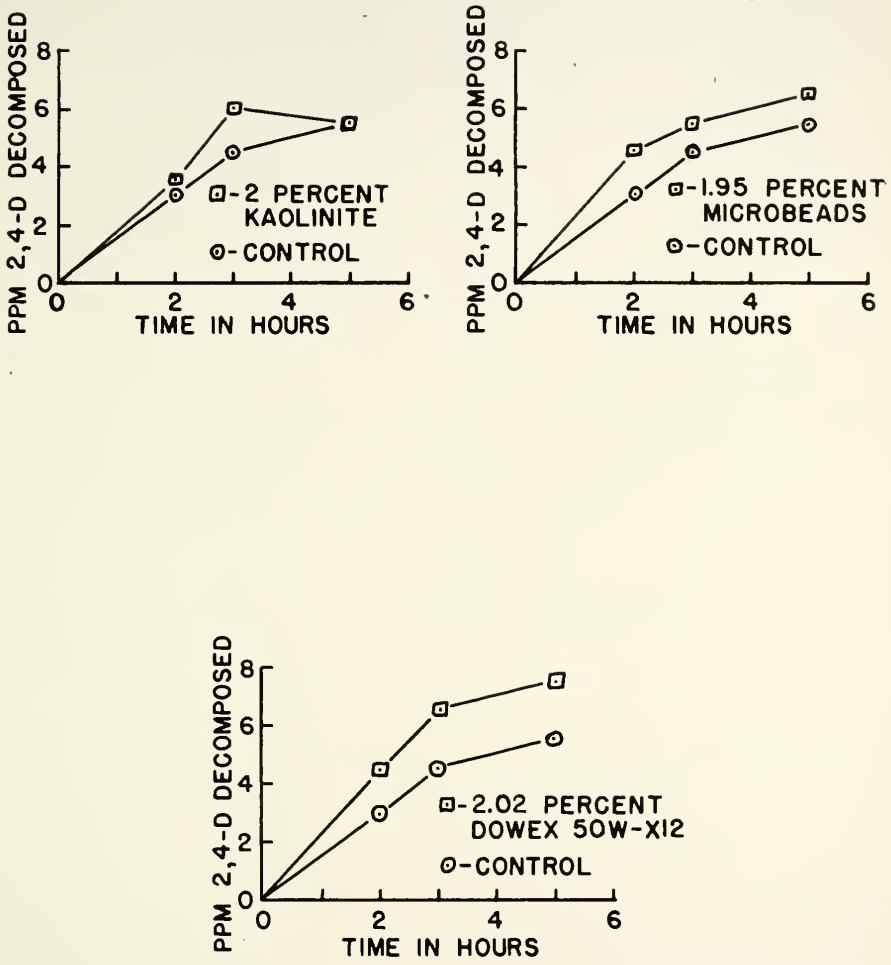
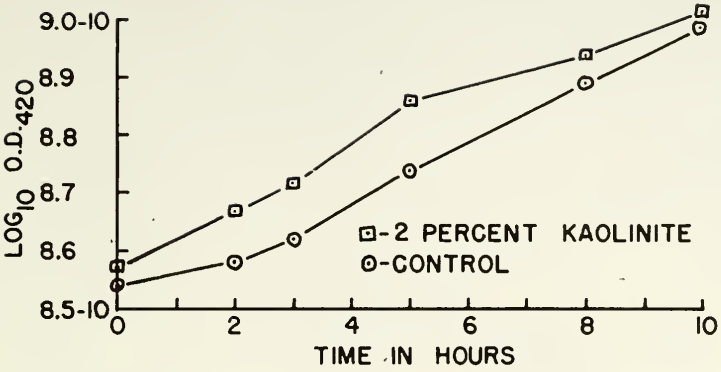


Figure 13. Effect of particles on bacterial decomposition of 10 ppm 2,4-D

may be expected. ZoBell (1946) postulated that bacterial adsorption on solid surfaces would prevent the rapid diffusion of exoenzymes from the bacterial surface. The resultant concentration of enzymes could have a beneficial effect on bacterial nutrition. Results of this study would indicate that this bacterium did not rely on exoenzymes for 2,4-D utilization.

Curve D in Figure 12 shows that there was no effect of the presence of Dowex 1-X8 on the uptake of 2,4-D by the bacterium until after 25 ppm 2,4-D had been decomposed. There was a decrease in the rate of 2,4-D decomposition when .1 percent Dowex 1-X8 was present after 25 ppm 2,4-D was decomposed compared with the rate when no resin was present. Apparently the 2,4-D, or at least a part of the 2,4-D, being held by the resin was inaccessible to the bacterium. The bacterium was decomposing the 2,4-D more rapidly than the resin was releasing 2,4-D into solution.

Bacterial growth on 2,4-D in the presence and absence of the particles was followed using turbidity measurements at the same time the 2,4-D uptake determinations were made. It can be seen from Figure 14 that the presence of kaolinite, Microbeads, Dowex 50 W-X12 and Dowex 1-X8 had no effect on the rate of bacterial growth in this study. These results were in agreement with those found for the effect of particles on the rate of 2,4-D uptake. The rate of bacterial growth as measured by turbidity is seen in



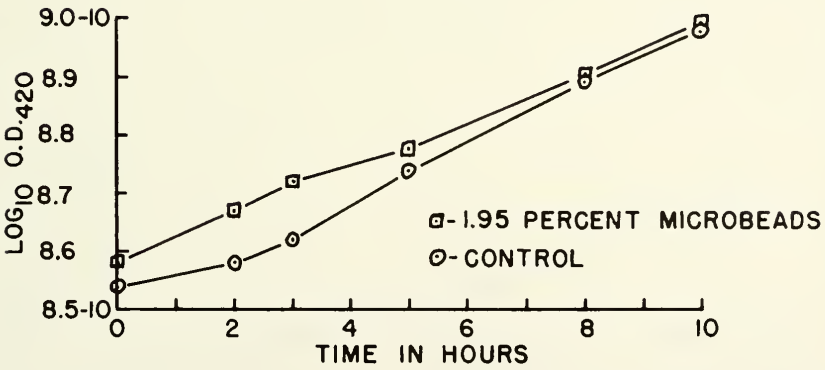
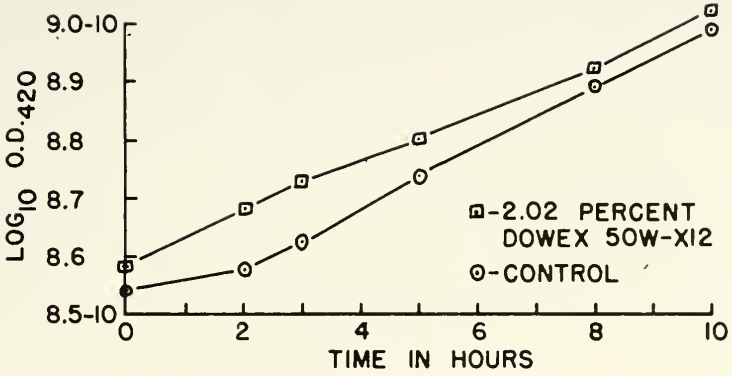
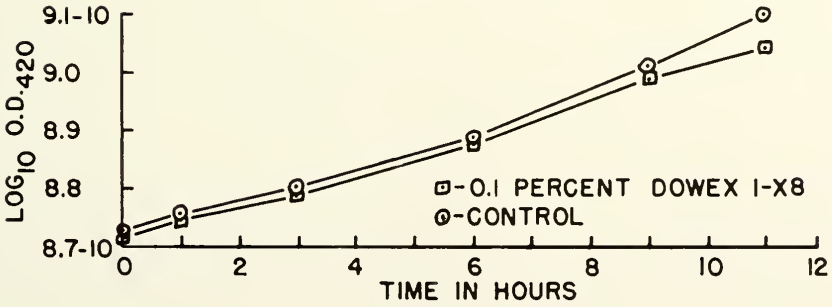


Figure 14. Effect of particles on bacterial growth in 100 ppm 2,4-D

Figure 14. (continued)





in Figure 15 not to vary in the range of 2,4-D concentration from 10 to 100 ppm which agrees with results obtained by viable counts in Part II above.

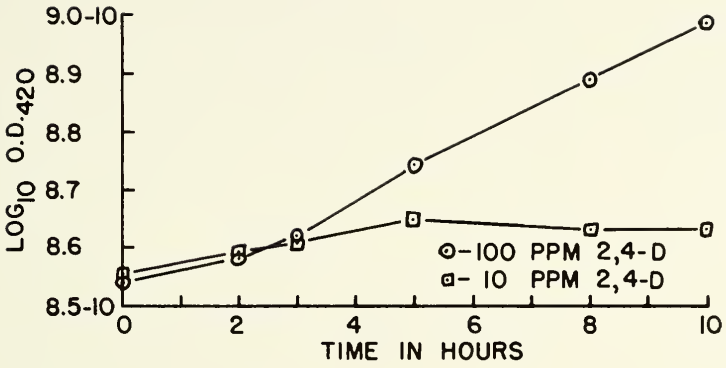


Figure 15. Bacterial growth curve in mineral salts medium containing 10 and 100 ppm 2,4-D

SUMMARY AND CONCLUSIONS

A system was developed as a model for elucidating some of the factors which affect the disappearance of herbicides in the soil. This system is applicable to the study of most herbicides for which organisms can be isolated which decompose the herbicide. In addition, the effects of other materials such as various clay minerals or lignin could be studied with this system. The following paragraphs enumerate the findings of a study using this system to investigate the effect of selected soil particles on the growth of a specific bacterium on a specific herbicide.

A bacterium capable of utilizing 2,4-D as a carbon source was isolated from the soil using the enrichment technique. Morphological and biochemical examinations of the bacterium showed it to have characteristics of a Pseudomonas. The decomposition of 2,4-D was accomplished by an induced enzyme system. Growth rate on 2,4-D did not vary in the range of 10 to 100 ppm 2,4-D.

The particles selected to simulate soil particles were washed by sedimentation and decantation. Microscopic examination was used to insure that the particles ranged in size from 8 to 149 μ in diameter. Dowex 50W-X12,

kaolinite, and Microbeads which possessed strong, weak and no cationic exchange capacity, respectively, did not adsorb 2,4-D from an aqueous solution at pH 7. Dowex 1-X8, an anion exchange resin, adsorbed 2,4-D moderately up to the anion exchange capacity of the resin.

Both kaolinite and Dowex 1-X8 adsorbed the bacterium indicating that the bacterial cell possessed positive and negative characteristics.

The presence of a two percent suspension of kaolinite, Microbeads, or Dowex 50W-X12 had no measureable effect on the rate of 2,4-D decomposition by a washed, heavy cell suspension of the bacterium. In addition, the initial rate of 2,4-D decomposition was unaffected by the presence of a 0.1 percent suspension of Dowex 1-X8. After 25 ppm 2,4-D was decomposed, there was a decrease in the rate when Dowex 1-X8 was present. The bacterium could not attack 2,4-D held by the resin and the 2,4-D in solution was utilized more rapidly than the 2,4-D was released by the resin.

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APPENDIX

Appendix Table 1. Decomposition of 120 ppm herbicide solutions in the percolation apparatus

Herbicide	Wavelength	Decomposition period
	mμ	days[1]
2,4-D	284	8
2,4-DP	284	85+[2]
4-(2,4-DB)	284	8
Trichlorophenylacetic acid	278	67+

[1] Reported as number of days required for 80 percent decomposition.

[2] + after a number indicates the herbicide was still present after the incubation period at the initial concentration.

Appendix Table 2. Calculations for Stokes' Law

Stokes' Law gives the rate of fall of a small sphere in a viscous fluid.

$$V = \frac{2ga^2(d_1-d_2)}{9\eta}$$

where V = velocity in cm per sec

g = force due to gravity in cm per sec^2

a = radius of sphere in cm

d_1 = density of sphere in grams per cm^3 = 2.60 for kaolinite

d_2 = density of medium in grams per cm^3

η = coefficient of viscosity in dyne-sec per cm^2 or poises = 0.008937 for water at 25°C

For kaolinite,

$$V = \frac{2(980 \text{ cm/sec}^2)\ a^2\ (2.60 \text{ g/cm}^3 - 1 \text{ g/cm}^3)}{9(.008937 \text{ dyne-sec/cm}^2)}$$

$$= 39000\ a^2/\text{cm sec}$$

For a kaolinite particle of 8 μ diameter,

$$V = 39000(4 \times 10^{-4})^2 \text{ cm}^2/\text{cm sec}$$

$$= 6.25 \times 10^{-3} \text{ cm/sec}$$

Thus in 26 min 40 sec all kaolinite particles 8 μ and larger in diameter would have fallen 10 cm. In the sedimentation process, all particles smaller than 8 μ were removed by siphoning the top 10 cm of fluid from a suspension of the particles after they had settled for 26 min 40 sec.